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DEVELOPMENTAL STUDIES AND LABORATORY INVESTIGATIONS

Conducted By Diagnostic Services National Animal Disease Laboratory Fiscal Year 1970

And Abstract of Published Report

Agricultural Research Service
U.S. DEPARTMENT OF AGRICULTURE

PREFACE

The project reports, abstracts of published reports, and case reports contained herein provide a reference source of the achievements made by Diagnostic Services, Animal Health Division, Agricultural Research Service, U.S. Department of Agriculture at Ames, Iowa, during fiscal year 1970 in this area of laboratory activity.

These reports are for the use of veterinary diagnosticians, epidemiologists, and regulatory officials in achieving their goals of providing increased diagnostic capabilities to the livestock and poultry industries.

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BRUCELLOSIS

A Serologic Survey for Actinobacillus seminis as the Etiologic Agent of Ram Epididymitis. Kelly, D. J., Brown, G. M., and Pietz, D. E. (Project Report).

Abstract

A survey was conducted in randomly selected flocks of sheep in Idaho to determine the significance of *Actinobacillus seminis* as the etiologic agent of epididymitis in rams. Complement fixation test results indicated that *A. seminis* was of little or no significance as the etiologic agent of epididymitis in these flocks.

Introduction

Workers in Australia[1] and South Africa[2] have described cases of ram epididymitis with *A. seminis* as the etiologic agent. *A. seminis* was first isolated in the United States in 1962 by Livingston and Hardy[3] from a Rambouillet ram.

A survey was being conducted in Idaho to determine the significance of *Brucella ovis* as the etiologic agent of ram epididymitis. Since rams were being palpated for clinical evidence of ram epididymitis, and blood samples were being collected from a random sampling of the rams with gross lesions of epididymitis, it was decided to utilize these blood samples to determine the significance of *A. seminis* as the etiologic agent of ram epididymitis.

Materials and Methods

Serums–Personnel of Biometrical Services, National Animal Disease Laboratory, Ames, Iowa, randomly selected 38 commercial Idaho flocks consisting of 1,000 or more ewes. All rams from these flocks were examined for gross lesions of epididymitis.[4] Eight rams from each flock were randomly selected for testing from those with gross lesions. If gross lesions were detected in less than eight rams, blood samples were collected from all rams with gross lesions plus a sufficient number of randomly selected rams without gross lesions to make eight rams per flock.

Control Serums–Positive antiserums were produced in Columbia rams artificially infected with *A. seminis*.[5] Negative antiserums were selected from ovine serums obtained from animals known to be free of *A. seminis*.

Complement-Fixation Antigen–*A. seminis* was inoculated on blood agar contained in Roux bottles and incubated at 37° C. for 48 hours. The growth was washed from the agar with sterile distilled water and boiled for 10 minutes. After filtering through gauze, the filtrate was titered against positive and negative serums and standardized against a control antigen.[5]

Complement-Fixation Test–Veronal buffer, complement, hemolysin and sheep red blood cells were obtained from Research Services, National Animal Disease Laboratory.

Hill's complement-fixation (CF) procedure[6] was followed with the following exceptions: (1) Serums were inactivated at 60° C. for 1 hour; (2) twofold dilutions of serum in veronal buffer were prepared (1:4-1:64); (3) 0.2 ml. of each serum dilution was used; (4) fixation time was 30 minutes; and (5) incubation time after the addition of sensitized sheep red blood cells was 30 minutes.

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[1]Baynes, I. D., and Simmons, G. C. 1960. Ovine epididymitis caused by *Actinobacillus seminis*, n. sp. Austral. Vet. Jour. 36(12): 454-459.

[2]Worthington, R. W., and Bosman, P. P. 1968. Isolation of *Actinobacillus seminis* in South Africa. Jour. S. African Vet. Med. Assoc. 39(2): 81-85.

[3]Livingston, C. W., and Hardy, W. T. 1964. Isolation of *Actinobacillus seminis* from ovine epididymitis. Amer. Jour. Vet. Res. 25(106): 660-663.

[4]The authors acknowledge R. E. Simmons, Idaho Sheep Commission, Boise, Idaho, for palpation for gross lesions of epididymitis and serum collection.

[5]The authors acknowledge G. C. Simmons, Animal Research Institute, Yeeronogpilly, Queensland, Australia for the *Actinobacillus seminis* culture and the control antigen.

[6]Hill, W. K. W. 1963. Standardization of the complement fixation test for brucellosis. Bull. Off. Int. Epizoot. 60: 401-417.

Results

A total of 303 serum samples were tested for complement-fixing antibodies. The results are recorded in table 1. Positive reactions were obtained on one and six serum samples at dilutions of 1:32 and 1:16 respectively.

Table 1.–Complement fixation test results for
Actinobacillus seminis

Serum end titer	Number of serums	Percent	
1:32	1	0.3	2.3
1:16	6	2.0	--
1:8	45	14.9	--
1:4	55	18.2	95.8
Negative 1:4	190	62.7	–
Not tested[1]	6	2.0	2.0

[1] Anticomplementary or quantity not sufficient.

Discussion

Although a significant titer for *Actinobacillus seminis* has not been determined, Simmons and others[7] and VanTonder and Bolton[8] considered a serum dilution of 1:20, with 50 percent or greater complement fixation, as positive.

In this study the rams were physically examined and selected for gross lesions of epididymitis. Therefore, a high proportion of those tested, 253 of 303 (85 percent), had gross lesions of epididymitis. There were only seven rams (2.3 percent) whose serums contained complement fixing antibodies at dilutions of 1:16 or greater and no serums contained antibodies at dilutions greater than 1:32. From these results it can be concluded that *Actinobacillus seminis* was of little or no significance as the causative agent for epididymitis in these flocks.

Evaluation of Antigens Prepared With Brucella Propagated in Liquid Medium. Pietz, D. E., Love, E. L., Andrews, M. G., and Johnson, K. P. (Project Report)

Abstract

Brucella abortus strain 1119-3 was propagated in liquid medium at 37° C. under constant agitation and aeration. The harvested suspensions were inactivated by heating at 80° C. for 1½ hours while being agitated in a Gyrotory water bath.

Antigens prepared with these suspensions of brucella were comparable in sensitivity to antigens prepared with brucella propagated on potato infusion agar and killed by heating to 95° to 100° C. for 1 hour.

All antigens used in the State-Federal Brucellosis Eradication Program are now being prepared with *Br. abortus* strain 1119-3 propagated in liquid medium.

Introduction

The objective of the State-Federal Brucellosis Eradication Program is the eradication of brucellosis from domestic animals. Since its inception in 1934, the basis of the program has been the detection and elimination of animal reservoirs by employing serologic test procedures.

[7]Simmons, G. C., Baynes, I. D., and Ludford, C. G. 1966. Epidemiology of *Actinobacillus seminis* in a flock of Broder Leicester sheep. Austral. Vet. Jour. 42: 183-187.

[8]VanTonder, E. M., and Bolton, T. F. W. 1968. Epididymitis in rams caused by *Actinobacillus seminis*. Jour. S. African Vet. Med. Assoc. 39(2): 87-90.

In 1909, Grinstead, a Danish veterinarian, was the first to use the tube agglutination test to detect brucellosis in cattle. (2)[1]

The serum plate agglutination test was developed by Huddelson and Carlson in 1926 (4). This test was called the rapid agglutination test for brucellosis as results, comparable to those in the tube test were obtained after an 8-minute incubation period as compared with 48 hours for the tube test.

In 1931, the United States Livestock Sanitary Association (USLSA), in an effort to obtain uniformity in the serologic diagnosis of this disease, recommended a uniform technique for conducting and interpreting the tube agglutination test for brucellosis (9). However, uniformity in results still remained a problem. Investigations conducted by the then Bureau of Animal Industry (BAI) revealed that variations in results were mainly caused by differences in the antigens used. Dr. Eichorn reported that variations in the sensitivity of brucella antigen could be caused by the media, dissociation, heat, preservatives, density, and the strain of brucella used (1). In an effort to remedy this problem, the American Veterinary Medical Association (AVMA), in 1938, recommended that the BAI prepare and furnish brucella antigen for the State-Federal Brucellosis Eradication Program (1).

Since 1939, all brucella antigen used in the State-Federal Brucellosis Program has been produced and distributed by the U.S. Department of Agriculture. Standard production procedures (6, 7) are used for the preparation of standard (tube, plate, and ring) and supplemental (rivanol and buffered brucella) test antigens.

All antigens have been prepared with *Brucella abortus* strain 1119-3 propagated on potato infusion agar, using standard procedures (5). The propagation of brucella on potato infusion agar is laborious and requires a large amount of equipment, such as laboratory carts and Roux flasks, and laboratory space. *Br. abortus* Strain 1119-3 has been successfully propagated in liquid medium (8) in this laboratory. The liquid medium method of propagation requires much less equipment, personnel, and space. Also, the process is a closed system and thereby gives laboratory personnel better protection from exposure to brucella.

A study was conducted to evaluate antigens prepared with brucella propagated in liquid medium by comparing their sensitivity with that of antigens prepared with brucella propagated on potato infusion agar. A report on the results of the study follows.

Materials and Methods

Brucella Suspensions – 1. *Br. abortus* strain 1119-3 was propagated on potato infusion agar, harvested, suspended, inactivated, and tested for sterility (5).

2. *Br. abortus* strain 1119-3 was propagated in liquid medium at 37° C., under constant agitation and aeration. The brucella was harvested, suspended, inactivated and tested for sterility (5), except that inactivation was accomplished by heating at 80° C. for 1½ hours and constantly agitating in a Gyrotory water bath.[2]

Brucella antigens – Antigens were prepared with suspensions of brucella propagated on potato infusion agar and in liquid medium according to the recommended standard procedures (6), (7). The following numbers of lots of antigens were prepared with suspensions of brucella propagated by each method: tube test antigen, 3 lots; plate test antigen, 3 lots; ring test antigen, 3 lots; rivanol test antigen, 2 lots; and buffered brucella antigen (BBA), 2 lots.

Sensitivity Determinations – 1. Tube test antigen. The six lots of antigen were coded and sensitivity tests conducted (6), except that 100 serum samples, with titers ranging from negative at 1:25 to positive at 1:2000, were tested. The total numerical reaction value for each antigen was determined.

2. Plate test antigen. The six lots of antigen were coded and sensitivity tests conducted as described (6) except that 100 serum samples were tested. The total numerical reaction value for each antigen was determined as described.

3. Ring test antigen. Serial dilution ring tests were conducted with each of the six antigens on five positive milk samples. The degree of reaction of each dilution of each sample was recorded, and the total reaction value for each antigen was determined by adding the reaction values of the five samples.

4. Rivanol test antigens. Sensitivity tests using the four lots of antigen were conducted as described, except that 50 serum samples were tested.

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[1] Underscored numbers in parentheses refer to references at the end of this Project Report.
[2] New Brunswick Scientific Company, Inc., New Brunswick, N.J.

5. Buffered brucella antigens (BBA). Each of the 50 serum samples was tested with the four antigens on the same card. After rocking for 4 minutes, the serum-antigen mixtures were visually observed for agglutination, and the degrees of reactions of the four antigens on each sample were compared.

Results

Tube Test Antigen—The total reaction value of the tube agglutination test results of 100 serum samples for each of the six lots of antigen is recorded in table 1. The average reaction value was 250.8 for the three lots of tube antigen prepared with brucella propagated on potato infusion agar and 249.8 for the three lots of tube antigen prepared with brucella propagated in liquid medium.

Table 1.—Tube agglutination test total reaction values on 100 serum samples.

Antigens prepared with brucella from potato infusion agar		Antigens prepared with brucella from liquid medium	
Lot No.	Total reaction value	Lot No.	Total reaction value
99	252.0	96	243.5
100	252.0	97	248.5
101	248.5	98	257.5
Average reaction value	250.8		249.8

Plate Test Antigen—The total reaction value of the plate agglutination test results of 100 serum samples for each of the six lots of antigen is recorded in table 2.

Table 2.—Plate agglutination test total reaction values on 100 serum samples.

Antigens prepared with brucella from potato infusion agar		Antigens prepared with brucella from liquid medium	
Lot No.	Total reaction value	Lot No.	Total reaction value
314	284.5	292	288.5
315	299.0	299	288.0
318	294.0	304	287.0
Average reaction value	292.5		287.8

Ring Test Antigen—The total reaction values of the serial dilution ring test of five positive milk samples for each of the six lots of antigen are recorded in table 3.

Table 3.–Serial dilution brucella ring test total reaction values for five positive milk samples

Antigen prepared with brucella from potato infusion agar		Antigen prepared with brucella from liquid medium	
Lot No.	Total reaction value	Lot No.	Total reaction value
80	149.0	81	148.5
82	149.0	84	149.0
83	148.0	87	149.0
Average reaction value	148.7		148.8

Buffered brucella antigen (BBA)–No differences were detected in the degrees of reactions obtained for each of the four lots of antigen on each of the 50 serum samples tested.

Discussion

In 1939, Eichorn (1) reported that medium on which brucella was propagated was a factor that contributed to differences in sensitivity of antigens prepared with brucella. Since the USDA began producing antigens for the State-Federal Brucellosis Eradication Program, this factor has been controlled by using a standard medium, potato infusion agar, for propagating *Brucella abortus* strain 1119-3. Although strain 19 vaccine has been prepared with brucella propagated in liquid medium, (3, 10, 11) antigens have not previously been prepared and evaluated. The results of this study indicate that tube, plate, ring test, rivanol, and buffered brucella antigens prepared with brucella propagated in liquid medium were comparable in sensitivity to the respective antigens prepared with brucella propagated on potato infusion agar.

According to the standard requirements, sensitivity determinations for all new lots of tube and plate antigens are conducted by using not less than 20 bovine serum samples that have titers ranging from negative at the 1:25 dilution to positive at the 1:200 dilution. These results are compared with the results obtained with standard tube and plate antigen respectively. A variation in sensitivity of plus or minus three dilutions is allowable, providing a difference no greater than plus or minus one-half dilution is obtained on any one sample. By the use of 100 samples, as was done in this study, a difference of plus or minus 15 dilutions would be allowable range. As shown in tables 1 and 2 (p. 4), all lots of tube and plate antigens were easily within this range.

In fiscal year 1970, four lots of tube and 21 lots of plate antigen were prepared with brucella propagated in liquid medium. The sensitivity of each of these lots was satisfactory when compared with the sensitivity of the standard antigen. All of these lots of antigens have been distributed and used in the State-Federal Brucellosis Eradication Program.

The results of this study also indicate that the sensitivity of ring test, rivanol test, and buffered brucella antigens were satisfactory when prepared with brucella propagated in liquid medium. In addition, during fiscal year 1970, seven lots of ring test and 23 lots of buffered brucella antigen were prepared with brucella propagated in liquid medium. The sensitivity of six of the seven lots of ring test antigen were satisfactory. All 23 lots of buffered brucella antigen were satisfactory in sensitivity.

The time and temperature used in killing brucella propagated in liquid medium affected the sensitivity of BBA. When brucella suspensions were heated to 95° to 100° C. for 1 hour, the BBA prepared was hypersensitive and when heated at 60° C. for 1½ hours, the BBA prepared was subsensitive. It was determined that satisfactory sensitivity was obtained when the suspensions of brucella were killed by heating at 80° C. for 1½ hours with constant agitation in a Gyrotory water bath.

All antigens used in the State-Federal Brucellosis Eradication Program are now being prepared with brucella propagated in liquid medium and killed by heating at 80° C. for 1½ hours in a Gyrotory water bath.

References

(1) Eichorn, A., and Thaller, H. I. 1939. Preparation of Brucella Antigen. Proc. U.S. Livestock Assoc., 43d Ann. Mtg.: 55.

(2) Grinstead, F. 1969. The Agglutination Test for the Diagnosis of Infectious Abortion in Cattle. Maanedsskr. Dyrl. 21: 395.

(3) Hauschild, A. H. W., and Pivnick, H. 1961. Continuous culture of *Brucella abortus* S. 19. Canad. Jour. Microbiol. 7: 491.

(4) Huddelson, I. F., and Carlson, E. R. 1926. A Rapid Method for Performing the Agglutination Test in the Serum Diagnosis of Bang's Abortion Disease in Cattle. Jour. Amer. Vet. Med. Assoc. 70: 229.

(5) NADL Diagnostic Reagents Manual 65A. The Propagation of *Brucella abortus* Strain 1119-3 for the Production of Brucella Antigens.

(6) NADL Diagnostic Reagents Manual 65B. The Production of *Brucella abortus* Standard Agglutination Test Antigens.

(7) NADL Diagnostic Reagents Manual 65C. The Production of Brucellosis Supplemental Test Antigens and Reagents.

(8) Pietz, D. E., and Love, E. L. (Unpublished data.)

(9) Report of the Committee on Bang's Disease. 1931. Proc. U.S.L.S.A., 35th Ann. Mtg.: 323.

(10) Sterne, M. 1958. The Growth of *Brucella abortus* Strain 19 in Aerated Dialysed Media. Jour. Gen. Microbiol. 18: 747.

(11) Van Drimmelen, G. C. 1955. Mass Cultivation of Micro-organisms with reference to Brucella Vaccine Against Contageous Abortion of Cattle. S. African Jour. Sci. 51:309.

Evaluation of Antigens and Strain 19 Vaccine Produced with Brucella Propagated on Medium Prepared with Dehydrated Potatoes. Love, E. L., Pietz, D. E., Andrew, M. G., and Johnson, K. P. (Project Report)

Abstract

Medium prepared with dehydrated potatoes was satisfactory for propagating *Brucella abortus* strains 1119-3 and 19 for antigen and vaccine production. Quality control tests indicated that the antigens and strain 19 vaccine prepared with brucella propagated on dehydrated potato medium were comparable to antigens and vaccine prepared with brucella propagated on potato infusion agar.

Dehydrated potatoes did not require as much storage space as raw potatoes, the low temperature-high humidity storage conditions were not necessary and less labor and equipment were needed for preparing the medium.

Dehydrated potato medium yielded slightly less brucella than potato infusion agar and it was not satisfactory for the selection of cultures.

Introduction

Potato infusion agar has been the standard medium for propagating *Br. abortus* strains 1119-3 and 19[1] for the production of antigens and strain 19 vaccine.[2] The use of this medium for the past 30 years has demonstrated that selecting and propagating these strains on this medium has been a satisfactory procedure for maintaining the antigenic and immunogenic qualities of these strains of brucella.

Although potato infusion agar is a satisfactory medium for propagating brucella, there are many disadvantages in using this medium. Sound, raw, well-cured potatoes are used for preparing the infusion. Ample storage space, at a temperature of about 40° C. and humidity of 85 percent is necessary for storing the potatoes. A considerable amount of labor and special equipment are required for washing, peeling, and slicing the potatoes. A large water bath is necessary for preparing the infusion by heating at 60° C. for 16 hours.

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[1] USDA, ARS, ANH Division. NADL Diagnostic Reagents Manual 65A. The Propagation of *Brucella abortus* strain 1119-3 for the production of brucella antigens. 1965.

[2] USDA, ARS, ANH Division. 1960. *Brucella abortus* strain 19 vaccine. ARS-91-24.

Even under ideal storage conditions potatoes deteriorate with age and often quantities are not suitable for preparing medium and must be discarded.

Dehydrated potatoes, in the form of flakes or flour, are now available. The use of dehydrated potatoes in lieu of potato infusion for preparing this medium would eliminate the disadvantages of storing and processing raw potatoes. A study was conducted to evaluate the growth of *Br. abortus* strains 1119-3 and 19 on medium prepared with dehydrated potatoes and the quality of antigens and strain 19 vaccine prepared with these brucella.

Materials and Methods

Potato infusion agar medium was prepared as recommended by the USDA.[3]

Dehydrated potato agar medium formula:

Potato flour	25 g.
or	
Potato flakes	30 g.
Agar, No. 1 granulated	20 g.
Peptone	10 g.
Beef Extract	5 g.
Sodium chloride	5 g.
Glycerine	20 ml.
Water, demineralized	1,000 ml.

Method of preparation: Five hundred ml. of demineralized water, in a 2 liter beaker, was heated to boiling. The potato flour or flakes were added slowly and stirred to prevent lumping. The peptone, sodium chloride, beef extract, and glycerine were added to the potato mixture and boiled until dissolved. This mixture was left at room temperature. The agar was added to 500 ml. of cold water and boiled until dissolved.

The two mixtures were combined and boiled, while being stirred until all ingredients were in the solution. The pH was adjusted to 7.0 by adding 10-percent sodium hydroxide. Approximately 125 ml. of medium was dispensed in each pyrex Roux flask. Gauze covered absorbent cotton plugs were used as closures. The Roux flasks containing medium were placed horizontally on a carrier. This was placed in an autoclave and sterilized at 121° C. for 30 minutes.

Propagation, harvesting, and preparation of antigens.—Br. abortus strain 1119-3 was inoculated, incubated, and harvested as recommended by the USDA.[4] Tube, plate, and ring test antigens were prepared using standard procedures.[5]

Propagation, Harvesting and Preparation of Strain 19 Vaccine.—Br. abortus strain 19 was inoculated, incubated, and harvested, and vaccine was prepared as recommended by the USDA.[6]

Results

Brucella antigens.—The quantity of *Br. abortus* strain 1119-3 harvested from the three types of medium is recorded in table 1. The average quantity of brucella harvested per Roux flask from the medium prepared with the flake type of dehydrated potatoes was somewhat less than from the medium prepared with the flour type of dehydrated potatoes (1.64 to 1.85 grams per Roux flask). The yield of brucella from the medium prepared with potato flour was comparable with the quantity of brucella harvested from potato infusion agar during the same period of time (1.85 to 1.89 grams per Roux flask).

[3] See footnote 1. p. 6.

[4] See footnote 1, p. 6.

[5] USDA, ARS, ANH Division. The production of *Brucella abortus* standard agglutination test antigens. NADL Diagnostic Reagents Manual 65B. 1965.

[6] See footnote 2, p. 6.

Less flour (25 g.) was required per liter of water than flakes (30 g.); however, the medium with flour was not as easily prepared as it was difficult to prevent the potato flour from forming lumps during mixing.

Table 1.—A Comparison of the Quantity of *Brucella abortus* strain 1119-3 harvested from the three types of media

Type of medium	Number of Roux flasks harvested	Grams of brucella harvested	Grams of brucella per Roux flask
Potato flour agar	5,260	9,733	1.85
Potato flake agar	2,957	4,839	1.64
Potato infusion agar	13,764	26,038	1.89

Four lots of tube antigen, five lots of plate antigen and one lot of ring test antigen were prepared with the brucella harvested from the medium prepared with dehydrated potatoes. Agglutination tests were conducted at 3- to 4-month intervals for 17 months to compare the sensitivity of these antigens with the sensitivity of antigens prepared with brucella harvested from potato infusion agar. Differences in sensitivity could not be detected.

Strain 19 Vaccine. — Two lots of liquid vaccine were prepared with *Br. abortus* strain 19 propagated on medium containing dehydrated potato flakes, and two lots of liquid vaccine were prepared with strain 19 propagated on medium containing dehydrated potato flour.

Colonial dissociative changes did not occur on either medium. The viability maintained during storage at refrigerator temperature for each of these vaccines was comparable to that of liquid vaccine prepared with strain 19 propagated on potato infusion agar.

Antigenic and virulency determinations were conducted with these vaccines and a vaccine prepared with strain 19 propagated on potato infusion agar by inoculating a standard dosage (3.0 X 10.9) of each vaccine into each of 12 guinea pigs. Differences in antigenicity and virulence were not detected.

One lot of lyophilized cultures was prepared with *Br. abortus* strain 19 propagated on medium prepared with flour dehydrated potatoes. Guinea pig responses produced by this lot of lyophilized cultures were comparable to those produced by lyophilized cultures prepared with strain 19 propagated on potato infusion agar. A lot of vaccine, prepared with seed cultures from this lot of lyophilized cultures, was satisfactory to all quality control tests.

Culture Selection.—Culture selection of *Br. abortus* strains 1119-3 and 19 by using reflected light[7] could not be performed with medium prepared with dehydrated potatoes. The dehydrated potato medium was much more opaque than potato infusion agar and reflected light would not pass through the medium. This was probably due to the large amount of starch in the dehydrated potato medium.

Discussion

The results of this study indicate that *Brucella abortus* strains 1119-3 and 19 can be propagated satisfactorily on medium prepared with dehydrated potatoes. Quality control tests conducted on antigens and vaccines prepared with brucella propagated on media containing dehydrated potatoes were comparable to the test results obtained on antigens and vaccines prepared with brucella propagated on potato infusion agar.

Using dehydrated potatoes to prepare medium for propagating brucella was a much more practical procedure than using raw potatoes for preparing potato infusion agar. Less storage space was required, low temperature-high humidity storage conditions were not necessary and less labor and equipment were needed for preparing the medium. However, dehydrated potato medium presented two problems: (1) The yield of brucella was not quite as high as the yield obtained from potato infusion agar, and (2) culture selection could not be performed with dehydrated potato medium. Therefore, it will not be possible to use this medium for maintaining and selecting cultures in this laboratory.

[7] See footnote 1, p. 6.

Dehydrated potato medium would be an ideal medium for commercial laboratories in this country or for laboratories in foreign countries involved in producing brucella antigens, strain 19 vaccine, or both. Seed cultures of strains 1119-3 and 19 are available from this laboratory; therefore, colonial selection is not necessary for these laboratories. At present, this medium can only be used as a backup procedure for propagating strain 1119-3 for antigen production in this laboratory because this strain is now being propagated in liquid medium. Potato infusion agar is necessary for the selection of cultures and it would be impractical to prepare dehydrated potato medium for the small amount of strain 19 vaccine produced in this laboratory.

Acknowledgment

Brown, G. M., Ranger, C. R., and Kelly, D. J. Selective Media for the Isolation of Brucella ovis. *Cornell Veterinarian.* [In Press.] *(Abstract of published report.)*

Thayer-Martin, a commercially available selective medium, was modified by eliminating IsoVitaleX, a chemically defined enrichment. The recovery rates of *Brucella ovis* from the semen of rams of this medium were increased significantly over recovery rates on tryptose agar enriched with 5-percent bovine serum.

Three groups of rams were used in evaluating the media in this study. Semen from two groups was cultured on three different media. Semen from a third group of rams was cultured on four different media. *Br. ovis* was isolated from a total of 138 semen samples. Of these, 127 (92 percent) isolations were made on Thayer-Martin medium without IsoVitaleX, and only 33 (24 percent) isolations were made on serum tryptose agar.

Of 11 failures to isolate on Thayer-Martin medium without IsoVitaleX, eight were attributable to an overgrowth of the medium by a swarming proteus-like contaminant. Further modifications were made in the medium to control this contaminant. The addition of furadantin prevented the growth of swarming bacteria in most instances; however, its inhibitory effect was overwhelmed when contamination was excessive. The addition of 2-percent ion agar No. 2 produced colonization in lieu of swarming. This medium was the most effective in preventing the growth of swarming bacteria; however, it had the disadvantage of being difficult to manage during preparation.

The use of a selective medium for the isolation of *Br. ovis* eliminated the need for extensive preparatory procedures in collecting semen samples. Therefore, the collection of semen for culture was facilitated, and the utility of this diagnostic procedure was markedly increased.

BOVINE VIRUS DIARRHEA

Evidence of Bovine Virus Diarrhea Infection in Colorado Antelope. Jenney, E. W., and Brown, C. L. (Project Report)

A serological survey of antelope serums was proposed to determine the susceptibility and potential role of wild antelope in the spread of bovine virus diarrhea (BVD) among cattle.

Material and Methods

Fifteen serum samples from each of six regions (see table 1) were submitted from 96 antelope killed during the 1968 hunting season in Colorado. The samplings included five to nine hunting areas within each region and were taken from 47 males, 40 females, and 9 sex unknown. No ages were given.

The serums were shipped frozen in dry ice to NADL and tested by a virus neutralization test using 100 1,000 TCID-50 of cytopathic BVD virus. Neutralization was carried out for 2 hours at 4° C. using a four fold serum dilution series. Either primary embryonic bovine kidney or bovine turbinate stationary-tube tissue culture (TC) was used for the neutralization tests. Cultures were incubated at 37° C. and read 96 hours after inoculation. Results

medium with 0.5 percent lactalbumin hydrolysate and 5-percent specific pathogen free calf serum was used as the TC medium. Endpoints were figured by the Karber-Spearman method with titers of 1.2 (the 1:16 serum dilution) considered to be strongly suspicious and above that as positive.

Results

Neutralization test results are summarized in table 1. One to two positive bovine virus diarrhea serums were found in six of the seven areas. There were two titers of 1.8, the 1:64 dilution, 2 of 2.1 (1:128), 2 of 2.4 (1:256) and 2 of 2.7 (1:512). Four animals with titers were males, three were females, and one was undetermined.

Table 1.—Summary of antelope-bovine virus diarrhea antibody survey (Colorado)

	Region	No. positive	No. negative	Total tested	Titer to BVD[1]
A	Northwestern Colorado	0	15	15	–
B	Northeastern Colorado	1	14	15	1.8 (1:64)
C	Northeast Central Plains	1	14	15	2.7 (1:512)
D	Southeast Central Plains	1	14	15	2.4 (1:256)
E	Southeastern Colorado	2	13	15	2.4, 2.1
F	Mountain valleys	1	5	6	2.7
G	Central Plains	2	13	15	1.8, 2.1
	Total animals	8	88	96	

[1] Titers are figured by the Karber-Spearman method and expressed as the logarithm of the serum dilution neutralized.

Conclusion

Eight percent of 96 antelope serum samples collected in Colorado during hunting contained bovine virus diarrhea antibodies ranging from 1.8 (1:64) to 2.7 (1:512). The positive animals were from six of seven regions' samples and involved equal percentages of males and females.

Evidence is presented indicating the susceptibility of the wild antelope population to bovine virus diarrhea. Infected antelope may contribute to the dissemination of bovine virus diarrhea virus between herds of cattle.

Acknowledgment

The authors gratefully acknowledge the technical assistance of Dennis Senne and John Love, Diagnostic Services, NADL, Ames, Iowa.

HOG CHOLERA

Isolation of Hog Cholera Virus from Tonsil Biopsies. Stewart, W. C., Kresse, J. I., and Carbrey, E. A. (Project Report)

Summary

Tonsil biopsies, taken from experimentally infected pigs with either a human rectal forceps or a drill-type, battery-operated instrument, were examined for hog cholera virus by the fluorescent antibody cell culture

technique. Virus was isolated from 25 of 34 specimens examined. In one case, virus was recovered before clinical signs appeared. Virus was isolated from seven pigs that had been sick for 10 or more days.

The practical application of isolating hog cholera virus from tonsil biopsies of febrile pigs in a natural outbreak of the disease was demonstrated.

Isolates recovered from tonsil biopsies were associated with the degree of virulence shown by the organism. Virus isolation was successful in 21 of 25 pigs infected with highly virulent strains but was successful in only four of nine pigs given strains of low virulence.

Introduction

When investigating the low virulent or less apparent type of hog cholera (HC), the diagnostician often has trouble determining which specimens to submit to the laboratory for diagnostic confirmation. Whole blood has been recommended when it is not feasible to necropsy a pig and collect the appropriate tissues. However, in a low-grade HC infection the development of an antibody titer in the serum results in the diminution or disappearance of virus from the circulation.

The fluorescent antibody technique has been employed to detect HC viral antigen in smears[1] and frozen sections[2] of tonsil biopsies. Tonsils of infected pigs have proved to be an excellent source of the virus.[3] In one study,[4] HC viral antigen was detected in the tonsils of pigs with low virulent infections when fluorescence was absent in other tissues.

This investigation was conducted to determine the possibility of isolating HC virus from tonsil biopsies of infected pigs.

Materials and Methods

Tonsil Biopsy Technique. — Tonsil biopsies were obtained either with a battery-operated, drill-type instrument (fig. 1) developed[5] specifically for swine or a Yeoman's human rectal forceps.[6] Pigs in a debilitated state were held in a caudal recumbent position by an assistant. A speculum was inserted into the mouth exposing the tonsils (fig. 2). Pigs that resisted were restrained with a snare.

The meager tissue fragments extracted with the mechanical drill were harvested by flushing 4 ml. of Earle's medium enriched with 5-percent specific pathogen free (SPF) calf serum over the cutting edge. Biopsies weighing 0.1 to 0.2 grams were obtained with the Yeoman's forceps and placed into small glass containers. In all cases, the biopsies were frozen immediately with dry ice and then transferred to a freezer at - 75° C. until examined.

Source of Biopsies. — Experimental infected, first and second generation, SPF pigs which ranged in weight from 30 to 50 pounds were arbitrarily selected as donors of the biopsies. The strains of HC virus employed to infect the pigs were the highly virulent Ames challenge strain and field strains of high and low virulence. The general condition of the pig was determined by the number of days of pyrexia or inappetence which occurred before collection of the biopsy.

In addition to the experimentally infected pigs, tonsil biopsies were received by the laboratory from three pigs out of a shipment of feeders moved from Missouri to Nebraska. The first death occurred 12 days after the movement, and when the investigation commenced 30 days after the shipment 17 of the 133 feeders had died.

Virus Isolation. — Tonsil biopsies obtained with the drill-type instrument were prepared for inoculation by macerating the tissue-fluid mixture in a Ten Broeck grinder. Biopsies taken with the Yeoman's forceps were ground

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[1] Nobuto, Kenzo. Personal communication, 1967.

[2] Teebken, D.L., Aiken, J.M., and Twiehaus, M.J. Differentiation of virulent, attenuated and inactivated hog cholera viruses by fluorescent antibody test. Jour. Amer. Vet. Med. Assoc. 150: 53-58. 1967.

[3] Carbrey, E. A., Stewart, W.C., Kresse, J.I., and Lee, L.R. Hog cholera virus assay of spleen and tonsil specimens. U.S. Agricultural Research Service, National Animal Disease Laboratory, Ames, Iowa. ARS 91-63, September 1967. 36 pp.

[4] Aiken, J.M., Hoopes, K.H., and Stair, E.L. Rapid diagnosis of hog cholera: a direct fluorescent antibody test. Proc. Amer. Vet. Med. Assoc.: 282-284. 1964.

[5] Kenzo Nobuto, Ministry of Agriculture and Forestry, Tokyo, Japan and Fujihira Industry Co., Ltd., Tokyo, Japan.

cians and Hospitals Supply Company, Inc., Minneapolis, Minn.
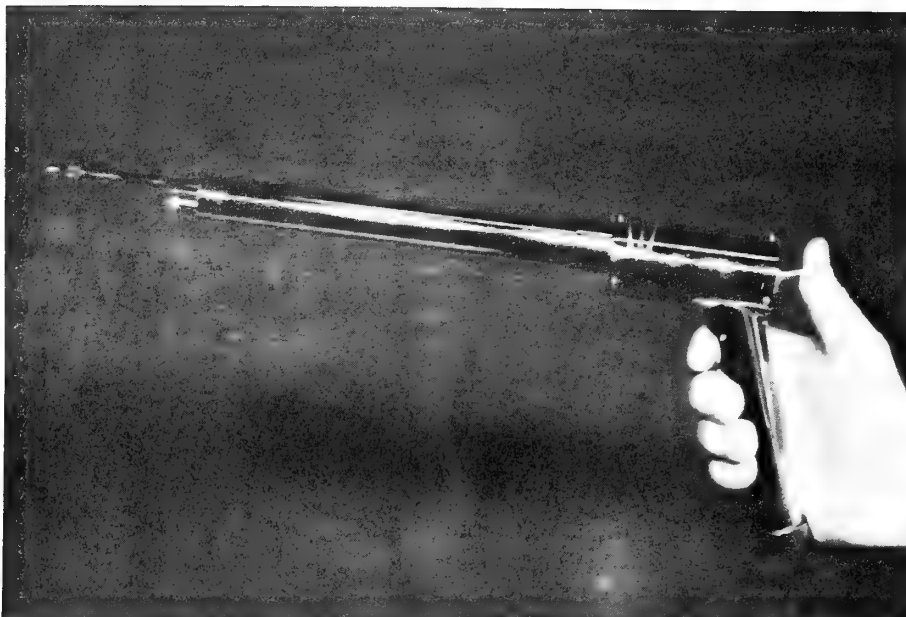
Figure 1.--Battery-driven, drill-type tonsil biopsy instrument.

Figure 2.--Tonsils exposed with the aid of a mouth speculum.

with a mortar and pestle. Suspensions of the tonsil biopsies were used to infect pig kidney (PK-15) cells and the fluorescent antibody cell culture technique (FACCT) was performed 24 and 48 hours postinfection as previously reported[7],[8]

Necropsies were performed on the pigs that succumbed to infection and the spleens were cultured for virus. In the final stage of the investigation, blood preserved in heparin was obtained concurrently with the tonsil biopsies, and the two specimens compared as a source of virus.

Virulence Determination. – Viruses designated as strains of high virulence caused illness which led to death of the test pig. Lesions of HC were observed at necropsy, and virus was detected in the tissues by the FACCT. Viruses considered to be strains of low virulence caused illness in the test pig which either recovered or died after a protracted course. In the case of death, lesions of HC were slight or absent and virus was not always recovered. If the pig survived, it was immune to challenge with virulent virus.

Results

Biopsies were taken with the Yeoman's forceps from 13 experimental infected pigs and distributed into five lots or composites consisting of one to five biopsies. Hog cholera virus was isolated from three of the composites. Subsequently, HC virus was isolated from the spleens of the pigs with two exceptions. In one instance a febrile reaction had prevailed only 3 days before euthanasia of the pig; in the other instance, were not cultured because the pigs resisted challenge with virulent (Ames) virus (table 1).

Biopsies were taken from 21 pigs with the drill-type instrument and examined by the FACCT. Hog cholera virus was isolated from the biopsies of 14 pigs. Subsequently, HC virus was isolated from the spleens of six pigs. Virus was not isolated from the spleen of one pig and spleens of the remaining seven pigs were not cultured (table 2).

Aspects of the clinical infection and recovery of virus from the tonsil biopsy were unusual in two pigs. Pig No. 4 (table 2) had apparently recovered after a rather protracted course of the disease. When challenged with virulent (Ames) HC virus 51 days postinfection, the pig had a relapse and died 15 days later. Serum, taken from this pig just before the administration of the Ames virus, contained HC virus, 1,340 fluorescent antibody infectious units per ml. This pig was considered chronically infected with HC at the time it was challenged with the Ames virus. Pig No. 14 (table 2) had recovered from infection and was also challenged with the Ames virus. Although the pig remained healthy to challenge, a tonsil biopsy taken 4 days later yielded HC virus.

Hog cholera virus was not isolated from the tonsil biopsies of five pigs infected with low virulent field strains and two pigs infected with the Ames strain of virus. In the case of the pigs infected with the low virulent strains, the biopsies were taken 1, 6, 17, 18, and 22 days after the onset of inappetence. Biopsies were taken from one pig infected with the Ames strain before inappetence occurred and from another pig 1 day after the onset of inappetence.

Table 1.–Isolation of hog cholera virus from tonsil biopsies taken with the Yeoman's forceps from experimentally infected pigs

Lot No.	Strain of virus- virulence	Average days pyrexia before collection of biopsies	No. of biopsies per lot	Fluorescent antibody infectious units per ml. of suspension	
				Tonsil biopsy	Spleen (Ave.)
1	Field-high	7.5	4	1,010	1,525
2	do	4	2	Neg.	[1]265
3	do	8	1	360	≥ 10,000
4	Ames-high	0	5	[2]Neg.	. . .
5	do	12	1	9	2,200

[1] Virus was isolated from the spleen of only one pig.
[2] Biopsies taken 3 days after challenge from pigs that had recovered from infection with a strain of low virulence.

[7]Carbrey, E.A., Stewart, W.C., Kresse, J.I., and Lee, L.R. Technical aspects of tissue culture, fluorescent antibody technique. Proc. U.S. Livestock Sanit. Assoc. 69: 487-500. 1965.

[8]U.S. Agricultural Research Service. Coverslip tissue culture, fluorescent antibody technique for the detection of hog cholera virus. Diagnostic Services, National Animal Disease Laboratory. 19 pp. 1968 (Mimeo.)

Table 2.–Isolation of hog cholera virus from tonsil biopsies of experimentally infected pigs taken with the drill-type instrument

Pig No.	Strain of virus-virulence	Days inappetence before collection of biopsies	Fluorescent antibody infectious units per ml. of suspension	
			Tonsil biopsy	Spleen
1	Ames-high	11	490	168
2	do	10	450	3
3	Field-high	10	42	470
4	Field-low	11	2	–
5	do	7	9	–
6	Field-high	5	3	Neg.
7	Ames-high	2	29	22
8	do	3	3	6,400
9	Field-low	17	8	–
10	Ames-high	4	141	54
11	do	13	1,000	–
12	do	7	2	–
13	do	6	8	–
14	do	0	1	–

Blood preserved in heparin and tonsil biopsies were taken simultaneously from 8 experimentally infected pigs. Hog cholera virus was isolated from all of the specimens (table 3). Hog cholera virus was subsequently recovered from the spleens of seven pigs infected with high virulent strains. One pig infected with a low virulent strain of virus recovered and later resisted challenge with the Ames strain 33 days postinfection.

Hog cholera virus was isolated from a composite of three tonsil biopsies received from the Nebraska swine herd. The fluorescent antibody infectious units per milliliter of tonsil biopsy suspension were 5,000.

Table 3.–Isolation of hog cholera virus from blood and tonsil biopsies taken simultaneously from experimentally infected pigs.

Pig No.	Strain of virus-virulence	Days inappetence before collection of specimens	Fluorescent antibody infectious units per ml. of inoculum	
			Blood	Tonsil biopsy
1	Ames-high	8	800	32
2	do	6	≥ 10,000	≥ 10,000
3	do	4	≥ 10,000	≥ 10,000
4	do	5	193	1,500
5	do	4	84	220
6	Field-high	2	≥ 10,000	250
7	Field-low	[1] 0	56	1,000
8	Field-high	2	≥ 10,000	240

[1] Biopsy taken 8 days postinfection.

Discussion

Thirty-four individual or composite tonsil biopsies were examined by the FACCT for HC virus. Virus was isolated from 25 of these specimens (73.5 percent). The fluorescent antibody infectious units ranged from a low count of one to 10,000 or more. Generally, the higher fluorescent antibody infectious units were obtained with the biopsies of pigs infected with strains of high virulence.

Of the 34 biopsies examined, nine were taken from pigs infected with strains of low virulence. The recovery rate of virus from these pigs was considerably less than that from pigs given strains of high virulence. Virus was isolated from only four of the nine biopsies, and the virus concentrations were low with three of the specimens.

Only four individual or composite tonsil biopsies taken from pigs infected with high virulent strains were negative for HC virus when examined by the FACCT. Of these four specimens, one composite of biopsies was taken from immune swine 3 days after challenge with virulent virus, one biopsy was taken from a pig before inappetence occurred, and a third biopsy was taken 1 day after the onset of inappetence. In the latter two cases the failure to isolate virus was attributed to the early stage of the infection.

However, HC virus was isolated both early and late in the course of the infection. Virus was recovered from the biopsies of pigs before clinical signs were evident in one case and as early as 2 days after the appearance of clinical signs in three others. Also, virus was isolated from seven pigs that had been ill for 10 or more days.

Hog cholera virus was isolated from blood and tonsil biopsies taken simultaneously from eight pigs. Of the six cases in which a distinction could be made, specimens with the greatest number of fluorescent antibody infectious units were equally divided between blood and tonsil biopsies. Two cases were not examined until 48 hours post-infection and the fluorescent antibody infectious units were too numerous to make a distinction.

Biopsies taken with the Yeoman's forceps were satisfactory for the isolation of HC virus by the FACCT. However, the forceps were developed for application in humans and, consequently, were cumbersome for use with swine. The forceps were also easily damaged. For these reasons the forceps were used sparingly in this investigation.

The battery-operated instrument was preferred for securing tonsil biopsies. The instrument had a small pen-light proximal to the bit end that facilitated location of the tonsils. The instrument removed a minimum of tissue thereby causing only slight pain and hemorrhage to the donor pig (figs. 3 and 4). However, the method required to harvest the biopsy from the bit end with tissue culture medium was a disadvantage of the instrument. A second disadvantage of the instrument was associated with the size of the biopsy. Its minute size precluded examination by the fluorescent antibody tissue section technique.

The isolation of HC virus from tonsil biopsies of naturally infected swine indicated that the method could be useful for application in the field.

Conclusion

The investigation confirmed that it was possible to isolate HC virus from tonsil biopsies of infected pigs by the FACCT. Virus was isolated from swine infected with high and low virulent strains, but the greatest success was achieved with the biopsies from swine infected with high virulent strains. The results indicated that total reliance should not be placed on tonsil biopsies in herds infected with a low virulent strain of virus. In addition, efforts should also be made to necropsy swine and submit the spleen, tonsils, and mandibular lymph node for virus culture.

Differences between tonsil biopsies and heparin preserved blood as a source of virus were minimal and consisted of only minor variations in the concentration of virus. However, to increase the virus recovery rate, both tonsil biopsies and heparin preserved blood should be taken from sick pigs when the owner is reluctant to permit euthanasia of swine for necropsy.
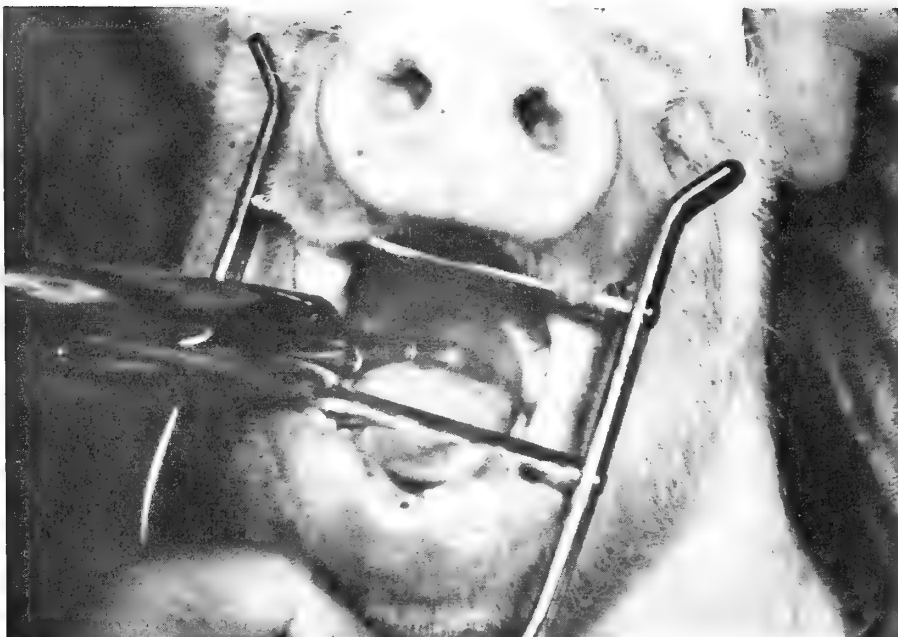
Figure 3.—Position of the instrument when a biopsy is taken.

Figure 4.—Small wound and minimal hemorrhage after removal of the biopsy.

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Carbrey, E. A., Stewart, W. C., Kresse, J. I., and Lee, L. R. Confirmation of Hog Cholera Diagnosis by Rapid Serum-Neutralization Technique. Amer. Vet. Med. Assoc. Jour. pp. 2201-2210. 1969. (Abstract of published report.)

A fluorescent antibody, serum-neutralization (FASN) test was developed and standardized for routine diagnostic use in the detection of serum antibody against hog cholera (HC) virus. Serum-neutralization titers were obtained by determining the highest serum dilution which caused a 90 percent reduction in the number of virus infectious units (VIU) on a coverslip cell culture. A test dose of HC virus of approximately 1,000 VIU was inoculated with each serum dilution on confluent PK-15 pig kidney cell cultures maintained in Leighton tubes.

Employing a fourfold dilution scheme, $\log_{10}$, serum titers of 1.8 to 3.0 (1:64 to 1:1,024) were detected at least 48 days following parenteral exposure of eight pigs to a field strain of HC virus of low virulence. The mean $\log_{10}$ titer was 2.3, which corresponded to an actual serum dilution of 1:200.

About 4 months after HC vaccination of 12 pigs as weanlings, $\log_{10}$ antibody titers of 1.2 to 2.4 (1:16 to 1:256) were obtained. After inoculation with virulent HC virus, the titer range of these pigs was 2.4 to 3.0. The mean postvaccination titer was 1.8, which increased to 2.6 following exposure of the pigs to virulent virus.

Serum titers were reported in pigs following ingestion of colostrum, exposure to bovine viral diarrhea virus, vaccination with inactivated virus, inoculation with antiserum, and short and long periods after exposure to HC virus.

Carbrey, E. A., Hesse, N. H., Stewart, W. C., Kresse, J. I., and Everson, E. E. The Filter Paper Disc Technique for the Detection of Antibodies Against Hog Cholera Virus. Proc. 73d Ann. Mtg. United States Animal Health Association, pp. 361-370. 1969. (Abstract of published report.)

A fluorescent antibody serum neutralization (FASN) test has been found a value in the diagnosis of hog cholera (HC) infection through the detection of serum antibodies against the virus. To facilitate the application of the FASN test, a technique was developed and standardized for collecting whole blood samples on filter paper discs. The blood samples were dried on the discs for convenient storage and shipment to the laboratories.

Only a small amount of blood was required, such as might be obtained from ear vein puncture. This method made bleeding from the anterior vena cava unnecessary with its attendant risks, requirements for careful restraint, and excitement of the animals which often creates unpleasant owner-veterinarian relationships.

Preliminary work with discs and blood specimens of known titer was performed. The eluate from a disc on which normal serum free of antibodies was dried slightly reduced the plaque count of the virus at the lowest dilution. This decrease was less than the 90-percent reduction required to indicate positive neutralization of this dilution. However, with blood samples containing HC antibodies, approximately the same neutralization titers were recovered from the paper discs as were found in serum samples collected at the same time.

A field study was instituted for the collection of bloods dried on filter paper discs and serums from the same pigs. A total of 162 duplicate specimens were collected from 29 herds where HC infection was suspected. No antibodies were detected in 83 blood-saturated discs and serums from 16 herds. The antibody titers for the remaining 79 specimens collected in the 13 serologically positive herds correlated quite well after a modification was made in the dilution scheme of the discs.

The filter paper discs have been employed to collect whole blood samples in Iowa, Florida, Alabama, North Carolina, Illinois, Nebraska and Tennessee. A total of 1,263 discs were submitted to the laboratory during a 6-month period. Some of the blood-soaked discs were from herds where HC infection was suspected. However, most of the discs were collected from wild swine where rugged field conditions made this method ideal for collecting and storing specimens for serology. Some case histories were presented where the disc specimens were of value in confirming or rejecting a diagnosis of HC.

It was suggested that the disc technique may be employed to advantage in the HC eradication program. Blood-soaked discs could be collected from 10 to 20 pigs on the initial visit to the farm. If the diagnosis of HC was not made quickly or was rejected, another set of blood discs could be collected from the same pigs on the last visit before the herd was released from quarantine 21 to 28 days later. Antibody titers to HC virus or rises in titers between the two bleedings would indicate infection with HC virus. On the other hand, the absence of HC antibody titers in the eluates from the discs would prove that HC infection had not been present.

SALMONELLA

Harrington, R., Ellis, E. M., Mallinson, E. T., Ranck, M., and Solee, R. E. An Evaluation of a Fluorescent Antibody Technique for the Detection of Salmonellae in Animal Byproducts, Feeds, and Tissues. Proc. 107th Ann. Mtg. of the Amer. Vet. Med. Assoc. (Abstract of published report.)

Four laboratories compared the fluorescent antibody technique (FAT) and standard methods for the detection of salmonellae in feeds, animal byproducts and swine tissues.

Of the 860 samples examined, 213 were positive by both methods, 240 were positive by FAT only, 20 were positive by culture only, and 387 were negative by both methods.

The FAT appears to be a rapid, highly accurate method for the detection of salmonellae in feeds, animal byproducts, and swine tissues.

TUBERCULOSIS

Rapid Confirmatory Test for Mycobacterium Paratuberculosis. Jarnagin, J. L., and Richards, W. D. (Project Report.)

Introduction

The lack of diagnostic tests has hindered the identification of *M. paratuberculosis*. The primary isolation of *M. paratuberculosis* is exceedingly slow on solid media and is dependent for growth upon mycobactin, an extract of *M. phlei*.

Because mycobacteria generally grow more rapidly in fluid than on solid media, Dubos broth containing mycobactin was used in this project in an attempt to reduce the growth time of *M. paratberculosis*. It was reasoned that the same cultural information regarding mycobactin dependency would be obtained in fluid medium as on solid medium but in less time.

The mycobactin used in the initial tests was a crude variety which produced a precipitate resembling bacterial growth. The precipitate interfered with the interpretation of results because it could not be differentiated from bacterial growth. The crude mycobactin was later replaced by the purer ferric mycobactin which did not cause the troublesome precipitate: Spectrophotometric measurements of growth were made possible by the use of purified ferric mycobactin.

Materials and Methods

Organisms.—The cultures of mycobacteria tested by this procedure were: *M. paratuberculosis* (70-03673-1052), *M. paratuberculosis* (70-03674-1053), *M. tuberculosis* (31507-935), *M. bovis* (70-01755-437), *M. avium* (28246-2307), Runyon Group II (Trudeau Strain P6), Runyon Group III (70-00460-101), Runyon Group IV (70-02728-713).

Test Procedure.—Cultures used in the test procedure were grown on Herrold's medium with mycobactin for 4 weeks before use. A portion of a colony from each culture was transferred with a 2 mm. loop and emulsified into 1.0 ml. of phosphate buffered saline (pH 7.0). Each culture was randomized and coded so the observer could not identify it until results were completed. Two drops of this suspension were inoculated with a pasteur pipette into each of six randomized 16- by 125- mm., screw-cap test tubes containing 5.0 ml. of Dubos broth fortified with Dubos Oleic Albumin Complex (DOAC) and Tween 80. Four of the six tubes contained 80 μg/ml. of ferric mycobactin and the remaining two tubes were controls and contained no mycobactin.

The bacterial density caused by the inoculum was determined spectrophotometrically in two tubes of fluid medium containing mycobactin and one without mycobactin. A spectrophotometer operating at a wavelength of 525 nanometers was used to make the determinations. The quantity of bacterial growth in the remaining three tubes of media was determined by gross observation of sedimented cells. The inoculated mediums were incubated at 37° C. for 4 weeks during which time the optical density and presence or absence of a growth sediment was determined every 48 hours.

Results

By the end of 14 days' incubation, maximal growth was reached by all mycobacterial cultures except the *M. paratuberculosis* cultures. The *M. paratuberculosis* cultures failed to grow during the four week incubation period. Results were identical for both the spectrophotometric and direct observation of growth sediment. The cultures of mycobacteria used and the spectrophotometric results obtained are summarized in table 1.

Table 1.–Spectrophotometric measurement of growth cultures of mycobacteria

Culture	Medium[1]	Days post inculation														
		2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
M. paratuberculosis 70-03673-1052	M	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	C	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M. paratuberculosis 70-03674-1053	M	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	C	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M. tuberculosis 31507-935	M	−	−	−	+	X	X	*	*	*	*	*	*	*	*	*
	C	−	−	+	+	X	X	X	X	X	X	X	X	X	X	X
M. bovis 70-01755-437	M	−	+	+	+	+	X	X	X	X	X	X	X	X	X	X
	C	+	X	X	X	X	*	*	*	*	*	*	*	*	*	*
M. avium 28246-2307	M	+	X	X	X	X	*	*	*	*	*	*	*	*	*	*
	C	X	X	X	X	X	*	*	*	*	*	*	*	*	*	*
Runyon Group II Trudeau P6	M	X	X	X	X	*	*	*	*	*	*	*	*	*	*	*
	C	X	X	X	X	*	*	*	*	*	*	*	*	*	*	*
Runyon Group III 70-00460-101	M	X	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	C	X	X	X	X	*	*	*	*	*	*	*	*	*	*	*
Runyon Group IV 70-02728-713	M	−	−	−	X	*	*	*	*	*	*	*	*	*	*	*
	C	X	X	X	*	*	*	*	*	*	*	*	*	*	*	*

[1]M = Medium containing mycobactin, C = Control medium without mycobactin, − = 91 to 100-percent transmittance, + = 60 to 90-percent transmittance, X = 30 to 59-percent transmittance, * = 0 to 29-percent transmittance.

Of the six different cultures which grew when tested by this procedure, not all grew at the same rate nor did all attain the same amount of growth. Despite variations in appearance time, all cultures with the exception of *M. paratuberculosis* eventually grew on all media.

Conclusion

The use of fluid medium containing mycobactin for the culture of *M. paratuberculosis* appears to offer no advantage over the use of Herrold's agar slants. An appearance time exceeding 4 weeks may account for the lack of growth in the fluid medium.

It is interesting to note that the *M. paratuberculosis* cultures did not grow during the test period while all other mycobacteria had reached maximal growth in 14 days. This selective growth pattern may be useful as an adjunct in differentiating *M. paratuberculosis* from all other mycobacteria.

Other investigators[1] have evaluated ferric mycobactin in fluid media and found that growth did not occur until 6 weeks after inoculation.

The cultures of *M. tuberculosis, M. bovis*, and Runyon Group IV that were tested appeared to be slightly inhibited or at least to have a delayed logarithmic growth phase by the presence of mycobactin in media. This factor should be considered in future work with a medium that contains mycobactin.

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[1]Merkal, R. S. Personal communication, April 1970.

The Production of Mycobactin, Pietz, D. E., Love, E. L., Johnson, K. P., and Andrew, M. G.
(Project Report)

Abstract

Mycobactin, an extract of *Mycobacterium phlei*, is an essential ingredient of media for the cultivation of *M. paratuberculosis* from the feces of cattle. Infected cattle are usually detected by this procedure before clinical signs are apparent. The mass production of mycobactin is necessary for this procedure to be a practical method for diagnosing *M. paratuberculosis*.

Laboratory procedures were developed for producing mycobactin at a reasonable cost and in sufficient quantities so as to make it available for use in the field.

Mycobactin is available to States where personnel have received proper training in the techniques for isolating *M. paratuberculosis*.

Introduction

Paratuberculosis (Johnes' disease) of cattle is a chronic enteric disease caused by *M. paratuberculosis* and is manifested by clinical signs of emaciation and diarrhea. Infected cattle without clinical signs usually perpetuate the disease in a herd. Various diagnostic procedures have been used for detecting cattle infected with *M. paratuberculosis*.

The intradermal johnin test has been the most common method used for testing herds of cattle suspected of having paratuberculosis. Larsen and others evaluated the intradermal test by conducting an extended study on a naturally infected herd of cattle (4).[1] The results indicated that (1) reactors to this test frequently become nonreactors, (2) not all infected cattle react to the test, and (3) many animals react intermittently to the test. Later reports on this same herd indicated that the complement-fixation and hemagglutination tests were not satisfactory for detecting infected cattle (3, 5).

Larsen and Kopecky reported that the intravenous johnin test was valuable for differentiating cattle with clinical paratuberculosis from those with other forms of chronic diarrhea, but was of little value in detecting lightly infected cattle (2).

The gel diffusion precipitin test was reported to be satisfactory for the diagnosis of paratuberculosis in sheep, but has not been evaluated in cattle (6).

The diagnosis of paratuberculosis by demonstrating the organisms by the microscopic examination of fecal specimens was reported to be of limited value because of the presence of other acid-fast organisms that were indistinguishable from *M. paratuberculosis* (8, 9).

Merkal and others determined the relative values of various tests for the early detection of paratuberculous cattle by comparing the results of the interdermal johnin test, gel diffusion test, complement-fixation test, microscopic examination of fecal specimens, and the cultural examination of fecal specimen (7). Cultural examination of fecal specimens detected infected animals from 1 to 2½ years before clinical signs were evident; therefore, this procedure was recommended for the early detection of paratuberculous cattle in infected herds.

Francis and others (1) reported on a growth factor called mycobactin, present in acid-fast bacteria, which was essential for the growth of *M. paratuberculosis*. Later Snow isolated the growth factor from *M. phlei* and called it mycobactin P, to distinguish it from related but chemically distinct growth factors from other species of mycobacteria (10). The author described procedures for extracting and assaying this growth factor from *M. phlei*.

Since cultural examination of fecal specimens was recommended for the early detection of paratuberculous cattle and mycobactin is essential for the growth of this organism, it was necessary to produce a sufficient quantity of this growth factor so that cultural examination could be used as a diagnostic procedure in the field.

The ANH Division requested that the Diagnostic Reagents Section, Diagnostic Services, National Animal Disease Laboratory (NADL), devise methods for producing sufficient mycobactin to meet these needs.

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[1] Underscored numbers in parentheses refer to Literature Cited at the end of this project report.

Materials and Methods

Mycobacterium phlei.—Cultures of *M. phlei* used for preparing the seed cultures were obtained from the Veterinary Sciences Research Division (VSRD), NADL, Ames, Iowa.

Medium.—Seed cultures of *M. phlei* were propagated in beef broth medium. Dorset and Henley's medium was used for the cultivation of *M. phlei.*

Equipment.—Roux bottles, carts and carriages for Roux bottles, a Lourdes blender, an Osterizer blender, and other miscellaneous equipment were already on hand in Diagnostic Services. Glassware, which included flat bottom pyrex flasks and Allihn condensers, were purchased. A ventilated drying oven was purchased and installed in the Diagnostic Reagents Section.

Preparation of Seed Cultures.—Beef broth medium was obtained in 100 ml. aliquots in 300 ml. Erlenmeyer flasks. A large loop, approximately 1/4-inch diameter, was used for inoculating. A loopful of *M. phlei* was removed aseptically from a previously prepared seed culture and placed on the surface of the beef broth by carefully submersing the loop into the medium. The seed cultures were incubated at 37.5° C. for 7 days.

Cultivation, harvesting, and drying of M. phlei.—The Dorset and Henleys medium was obtained in 120 ml. aliquots in Roux bottles that were closed with cotton plugs wrapped with gauze. Carriages, each holding 216 Roux bottles, on carts were used for transporting the Roux bottles of media in the laboratory.

Seed cultures were blenderized in a Lourdes blender and approximately 0.5 ml. of the "foamed" organisms and medium were inoculated, using a 30 ml. syringe and a 6-inch, 12-gage needle, on the surface of the media of each Roux bottle. Three carts of Roux bottles were inoculated each week. Incubation was at 37.5° C. for 10 days. The media was removed from the *M. phlei* by filtering through cotton gauze. Organisms that remained clinging to the surface of the Roux bottles were removed by rinsing with distilled water. After the media was drained off, the organisms were rinsed with distilled water and allowed to drain thoroughly in a refrigerator overnight. The organisms were then dried at 75° C. for 3 days in a ventilated oven. The dried organisms were ground to a fine powder with an Osterizer blender.

Extraction of Mycobactin.—1. Acetone extraction. Four hundred grams of dried *M. phlei* were put into a 6-liter, flat bottom, pyrex flask, and 2 liters of acetone added. An Allihn condenser was attached to the flask, and tap water was circulated through the condenser. The mixture was refluxed for 30 minutes by heating and mixing on a "King Size" magnetic stirrer using a 2½-inch, teflon encased, magnetic stirring bar. The organisms were allowed to settle, and the acetone extract was poured off into an amber bottle. These acetone extraction procedures were repeated three more times, using 2 liters of acetone each time. The acetone extracts were labeled extracts 1, 2, 3, and 4. Acetone extracts 1 and 2 were cooled, filtered through S and S 520-B-½ high-speed paper and evaporated in a ventilated oven. Acetone extracts 3 and 4 were stored in amber bottles in a refrigerator and used for performing extractions 1 and 2 of the next batch of *M. phlei.*

2. Methanol extraction. The residue obtained by drying acetone extracts 1 and 2 of 10 quantities (4,000 grams) of powdered dried *M. phlei* were put into a 1,500 ml. beaker, and approximately 1 liter of methanol added. This was placed on a King Size magnetic stirrer contained in a fume hood, a 2½-inch teflon encased magnetic bar added, and the mixture stirred and boiled until all residue was dissolved. This material was cooled overnight in a refrigerator and filtered through S and S 520-B-½ high-speed paper. This methanol extraction procedure was repeated on the residue collected in the filter paper until the methanol was clear after being cooled overnight in a refrigerator. The methanol extracts were combined and evaporated to dryness at 75° C. in a ventilated oven.

3. Petroleum ether extraction. The dried methanol extraction residue was put into a beaker and mixed with 2 to 3 volumes of petroleum ether on a magnetic stirrer. The residue was allowed to settle to the bottom, and the petroleum ether was poured off into a beaker. This procedure was repeated until the petroleum ether was clear. The petroleum ether poured from the residue was evaporated to a small volume (approximately 100 ml.) at 75° C. in a ventilated oven and centrifuged. The petroleum ether was poured off, and the residue was washed with petroleum ether by centrifugation until the petroleum ether was clear. All of the petroleum ether extraction residue was put into an evaporating dish, stirred for 30 minutes at room temperature, and dried in a 37° C. incubator overnight.

4. Boiling water extraction. The dried petroleum ether extraction residue was put in a high speed filter paper, and boiling water was poured over the residue until the water was clear. The residue was evaporated to dryness at 75° C. in a ventilated oven. The dried residue was removed from the filter paper with petroleum ether and evaporated to dryness at 75° C. in a ventilated oven. This final residue was mycobactin.

Bottling and Labeling of Mycobactin.—The mycobactin residue was weighed and mixed with absolute ethyl alcohol at a ratio of 150 mg. per 4 ml. of alcohol. This mixture was heated and mixed on a magnetic stirrer until the mycobactin was in solution. Using a 5-ml. rheometer syringe, 4 ml. of the solution was delivered into each 4-dram, wide-mouth vial. The mycobactin was precipitated by cooling in a refrigerator, and the alcohol was evaporated in a 37° C. incubator. The vials were labeled with the name of the product, quantity contained, and the production date.

Quality Control.— Mycobactin was dissolved in methanol (0.02 mg./ml.) and checked for purity by determining the absorbency curve on a spectrophotometer. A liter of modified Herrold's Egg Yolk Medium was prepared with 150 mg. of each new lot of mycobactin. This media was compared with media prepared with a previous lot of mycobactin by cultivating *M. paratuberculosis* on each media.

Distribution of Mycobactin.—ANH Division Memorandum 587.24, dated July 16, 1969, gives the Division policy for the culture of *M. paratuberculosis*. This policy limits the quantities of mycobactin that will be supplied to States requesting it, providing the requesting State has laboratory personnel properly trained in the techniques for isolating *M. paratuberculosis*. Such personnel training is available in the Mycobacteriology Section of Diagnostic Services, ANH Division, NADL, Ames, Iowa. This training must be authorized by and arranged for through the appropriate assistant director.

Results

During fiscal year 1969, over 7,000 Roux flasks of Dorset and Henleys medium were inoculated with *M. phlei*, and 9,695 grams of dried organisms were harvested. Mycobactin was extracted from 7,179 grams of dried *M. phlei*, yielding 63,450 mg. of mycobactin. This quantity of mycobactin was sufficient for preparing 423 liters (approximately 42,300 tubes) of Herrold's Egg Yolk Medium.

Mycobactin was dispensed in 150 mg. quantities, which is the quantity required per liter of medium. During fiscal year 1969, 30,300 mg. of mycobactin were distributed to the VSR Division at NADL, the Diagnostic Bacteriology Section at NADL, and the States of Wisconsin and Illinois.

Discussion

A growth factor called mycobactin, which is extracted from mycobacteria such as *M. phlei*, is an essential ingredient of media for the cultivation of *M. paratuberculosis*. Culturing cattle fecal specimens for *M. paratuberculosis* has been shown to be an effective method for detecting infected cattle before clinical signs are apparent. Field work is necessary to more thoroughly evaluate the effectiveness of this method for eliminating *M. paratuberculosis* from infected herds. However, this procedure would not be practical unless mycobactin can be produced and distributed in large quantities at a reasonable cost.

Various time-saving modifications were implemented in this study in an effort to produce this product in large quantities and at a reasonable cost.

One large lot (85 liters) of Dorset and Henleys medium was prepared each week. Therefore, approximately 700 Roux bottles of medium were inoculated and harvested each week.

The dried *M. phlei* was ground to a fine powder with an Osterizer homogenizer rather than with a mortar and pestle.

Acetone extraction was performed on large quantities (400 grams) of dried *M. phlei*, and acetone extracts 3 and 4 were used for extractions 1 and 2 of the next quantity of *M. phlei*. The balance of the extraction procedures were conducted on 10 batches of acetone extraction residue.

Dispensing was accomplished by dissolving the mycobactin in absolute ethyl alcohol and delivering into each vial with a rheometer syringe rather than weighing 150 mg. quantities.

Although only 63,450 mg. of mycobactin were processed during fiscal year 1969, an estimated 250,000 mg. could be produced using these procedures on a full-time basis. This would be sufficient for preparing 1,667 liters (166,700 tubes) of Herrold's Egg Yolk Medium.

These procedures have made it possible to produce mycobactin at an estimated cost of $75.00 per gram. This would amount to approximately $1.00 per tube of Herrold's Egg Yolk Medium.

This study has demonstrated that mycobactin can be produced in sufficient quantities for diagnostic purposes. States wishing to conduct fecal culturing for isolating *M. paratuberculosis* can obtain mycobactin upon request. However, it is necessary that the requesting State has laboratory personnel who have received proper training in the techniques for isolating this organism. Such training is available in the Mycobacteriology Section of Diagnostic Services, ANH Division, NADL, Ames, Iowa. This training must be authorized by and arranged for through the appropriate assistant director.

Acknowledgment

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The Use of calves in the Bioassay of Purified Protein Derivatives. Richards, W. D. (Project Report)

Introduction

Purified protein derivatives (PPD) of mycobacterial origin were produced in compliance with developmental project requirements. Each product was tested for purity, sterility, chemical composition, and allergenic sensitivity in guinea pigs. The allergenic specificity was not generally determined.

The experiment described here was conducted to determine the sensitivity and specificity of *Mycobacterium bovis* and *M. avium* PPD's in cattle infected with the homologous and heterologous mycobacteria.

Data regarding the specificity of PPD's were needed to evaluate them for use in comparative skin tests. The mycobacterial species causing infection is indicated, in some instances, by comparing the host responses to two or more skin test agents injected simultaneously (4,5).[1]

Materials and Methods

*Animals.—*A holstein steer, No. 1, was acquired from the specific pathogen free (SPF) herd at the National Animal Disease Laboratory. It weighed approximately 150 pounds when acquired at 10 weeks old. It had never been tuberculin-skin tested. It was inoculated intratracheally (IT) with 1.0 ml. of a suspension of *M. bovis* strain ATCC 19211 containing 0.1 mg. per ml. (wet weight) of virulent cells. At 10 weeks before inoculation, the calf was tuberculin-skin tested with 21 different tuberculins and PPD preparations and one buffer by intradermal injections in the clipped cervical region. No two test sites were less than 6 inches apart. Two of the same test agents were injected ID into the caudal folds; Agricultural Research Service (ARS) Reference Old Tuberculin (OT) in the left fold and Netherlands *M. bovis* PPD in the right fold. The calf was necropsied after the skin test responses were read.

Calf No. 2 was the same as calf No. 1 regarding origin, age, weight and tuberculin skin test history. It was inoculated IT with 0.1 ml. of a suspension of *M. bovis* strain ATCC 19211 containing 0.1 mg. per ml. (wet weight) of virulent cells. The skin tests administered to the cervical region and caudal folds were the same as in calf No. 1. The calf was necropsied after the skin test responses were read.

Calf No. 3 was a holstein steer acquired from a local dairy in which no tuberculosis had ever been found by routine surveillance practices. The steer had never been tuberculin skin tested. It weighed approximately 210 pounds when acquired at 4 months old. It was exposed to chickens infected by intraperitoneal (IP) injection with a virulent *M. avium* culture isolated from swine. The chickens and calves were free to move about in a 12-foot by 12-foot room. They ate and drank from common feed and water stations. The exposures continued for 23 weeks.

A total of 25 skin test agents and one buffer control were injected ID in the cervical region 22 weeks after exposure was started. ARS reference OT was injected in the left caudal fold and Netherlands *M. bovis* PPD was injected in the right caudal fold. The calf was necropsied 23 weeks after exposure to tuberculous chickens was begun.

Calf No. 4 was from the same farm as calf No. 3. It weighed approximately 250 pounds when acquired at 5 months of age. It was exposed to the same tuberculous chickens as was calf No. 3. The same skin test agents were administered and the calf was necropsied 23 weeks after exposure to tuberculous chickens was begun.

Ten white Leghorn chickens were approximately 4 weeks old when placed in the animal room with calves No. 3 and No. 4. Four of the chickens were inoculated IP with 1.0 ml. of a 0.05 mg. per ml. (wet weight) suspension of virulent *M. avium* cells and placed in the animal room at the beginning of the experiment. Two chickens were similarly treated except that the concentration of the inoculum was 0.1 mg. (wet weight) of cells per ml. Two uninoculated chickens were placed in the animal room at the beginning of the experiment and two other healthy chickens were placed in the animal room approximately 1 month after the experiment was begun.

Cultures.—M. bovis strain ATCC 19211 was isolated from tuberculous bovine tissue. The subculture for this experiment was grown in Dubos Broth for approximately 3 weeks at 37° C. The sedimented cells were resuspended in Butterfield's Buffer to a concentration of 0.1 mg. per ml. (wet weight of cells).

The *M. avium* culture (accession No. 28246) was isolated from tuberculous swine tissue. The culture for this experiment was prepared by the same methods described for *M. bovis* ATCC 19211.

*Skin Test Agents.—*The PPD test agents number "pre," 1, 2, 3, 4, and 5 were prepared from the culture filtrate of *M. bovis* 110, a stock culture obtained from Canadian Department of Agriculture, Hull, Quebec, Canada, grown on Reid's synthetic medium.

PPD-6 was prepared from *M. bovis* strain AN 5, a stock culture obtained from the Ministry of Agriculture Fisheries and Food, Weybridge, England, grown on Long's Synthetic Medium. The PPD of *M. avium* origin was prepared from the USDA stock strain St. Elizabeth (stE). The PPD of *M. avium* prepared in Weybridge, England, was produced from strain D 4. The PPD preparations which were precipitated by trichloroacetic acid were designated "TCA." Those precipitated by ammonium sulfate were designated "AS."

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[1] Underscored number in parentheses refer to references at the end of the project report.

The Purified Protoplasmic Peptide (PPP) (1) was prepared from *M. bovis* strain BCG at the Communicable Disease Center, Atlanta, Ga. It was designated "PPP–BCG."

Veterinary Biologics Division reference Old Tuberculin (OT) number 11 was used as a skin test agent. It was produced for the USDA in accordance with contract requirements. Culture filtrates of *M. tuberculosis (M. tb)* strains C, Dt, and Pn grown on synthetic medium were concentrated by heat (HCSM).

Results

The results of 100 injections of skin test agents and four injections of control buffer are shown in table 1. Only two homologous and five heterologous tests failed to give the expected response. Five cross reactions occurred and all were by PPD precipitated with TCA. No response was present in calf No. 2 at the site of injection of two PPD's: PPD 4 TCA produced at NADL and PPD 110 as produced by the Canadian Department of Agriculture. The Old Tuberculin (HCSM) of *M. tuberculosis* origin and *M. bovis* PPD injected into the caudal folds of calves 1 and 2 caused positive responses. There was no response to the same agents injected into the caudal folds of calves 3 and 4.

Table 1.–Sensitivity and specificity of tuberculin-skin tests in cattle

Test agent	Precipitation method	Myco species	Source	Dose mg./0.1 ml.	Response[1] M. bovis infected calves		M. avium exposed calves	
					1	2	3	4
PPD-Pre	TCA	*M. bovis*	USDA, NADL	0.65	10A	35A	40C	--
PPD-1	TCA	do	do	.91	20B	10B	15A	–
PPD-1	AS	do	do	.61	25C	15C	--	--
PPD-2	TCA	do	do	.44	20A	25B	--	--
PPD-2	AS	do	do	.20	25C	20B	–	--
PPD-3	TCA	do	do	1.43	20A	20A	15A	--
PPD-3	AS	do	do	1.44	25C	20B	--	--
PPD-4	TCA	do	do	1.12	10A	–	--	--
PPD-4	AS	do	do	.64	25C	15A	–	--
PPD-5	TCA	do	do	.32	(2)	(2)	10A	--
PPD-5	AS	do	do	.54	(2)	(2)	–	--
PPD-6	TCA	do	do	.37	(2)	(2)	--	--
PPD-6	AS	do	do	.26	(2)	(2)	--	--
PPD-AN5	TCA	do	Netherlands	.10	25C	25B	--	--
PPD-AN5	TCA	do	England	.10	20B	25B	–	--
PPD-AN5	TCA	do	S. Africa	.14	20C	30B	--	--
PPD-110	AS	do	Canada	.10	20C	–	--	--
PPD-110	TCA	do	do	.10	25B	30C	--	–
PPD-110	T&A[3]	do	do	.10	20C	20A	--	–
PPP-BCG	N/A	do	USPHS, CDC	.01	5B	10A	–	–
PPP-BCG	N/A	do	do	.10	15C	10B	–	–
HCSM-11	OT	M. tb.	USDA, ARS	.29[4]	35A	20A	--	--
PPD	TCA	*M. avium*	USDA, NADL	.67	--	--	45C	45C
PPD	AS	do	do	.28	--	–	35B	35B
PPD	TCA	do	England	.05	--	15A	30B	25A
Control-Phenolized phosphate buffer. 0.1 ml. I.D.					--	--	--	--

[1] Numerical response is the greatest diameter of induration expressed in millimeters. The letters designate the protrusion of the induration: A = 3 mm. or less, B = 4 to 6 mm., C = 7 mm. or greater, – = no response.

[2] No test. PPD-5 and PPD-6 were not available.

[3] A mixture of equal parts of PPD precipitated by TCA and AS methods.

[4] Amount of protein estimated from contract requirement of 0.047 percent TCA precipitable nitrogen.

Calf No. 1 was tuberculous at time of necropsy. A caseous abscess was present on the trachea at the site of inoculation. Bronchial lymph nodes were enlarged and contained a caseous exudate. Extensive miliary tubercles were observed in the lungs. The histopathology report on lung tissue was "Compatible for Mycobacterioses." *M. bovis* was isolated from lung lesions.

Calf No. 2 was tuberculous at time of necropsy. A caseous abscess was present on the trachea at the site of inoculation. Bronchial lymph nodes were enlarged and contained caseous exudate. The histopathology report was "Compatible for Mycobacterioses." *M. bovis* was isolated from bronchial lymph nodes.

The mesenteric lymph nodes of calf No. 3 were enlarged and hemorrhagic at necropsy. The ileocecal valve and a small section of the small intestine were also hemorrhagic. The lungs were pneumonic. The histopathology report on mesenteric lymph nodes was "compatible for mycobacterioses." *M. avium* was isolated from the mesenteric lymph nodes.

Calf No. 4 had approximately the same pathologic changes as calf No. 3 except for some calcification and more enlargement of mesenteric lymph nodes. The histopathology report was "compatible for mycobacterioses" and *M. avium* was isolated from mesenteric lymph nodes.

Tuberculous lesions were present in the liver, spleen and intestine of the six inoculated chickens. *M. avium* was isolated from the liver of five of them. The issues of the sixth inoculated chicken was not cultured. *M. avium* was isolated from the feces of three inoculated chickens. No isolation was made from the fourth chicken and no culture was attempted on the fifth and sixth chickens. There was no evidence of tuberculosis at necropsy in one of the two healthy chickens placed in the animal room 1 month after the exposure period was begun. No isolation was attempted from tissues. Lesions were present and *M. avium* was isolated from the liver and spleen of the other chicken placed in the animal room 1 month after the exposure period was begun. No isolation was made from the feces of either of the two chickens.

Discussion

The specificity of various mycobacterial skin test agents was confirmed when 97 of a possible 104 responses were compatible with the expected results in calves infected with the homologous and heterologous organism. Two of the undesired responses were obtained by the injection of test agents produced in foreign countries. The four cross responses in calf No. 3 (infected with *M. avium*) were caused by PPD preparations made in this laboratory by the TCA precipitation method. It should be noted that the dose of one of the four was 6.5 times the average dose of 0.1 mg. The other three were 9.1, 14.3, and 3.2 times the average dose. The doses were not standardized because the chemical assay was not completed at the time calves No. 1 and No. 2 were tested. The test agents were standardized by the time calves No. 3 and No. 4 were tested, but it was considered more important to administer the exact agents used in calves infected with *M. bovis*.

Conflicting reports (2,3,6,7) have been published regarding the alteration of cutaneous sensitivity in individual animals injected simultaneously with two or more tuberculins. No cattle received single injections of tuberculin in this experiment as controls so no accurate measure of the alteration of sensitivity was made. It was concluded, however, that allergic interaction of tuberculins was not appreciable because large responses were obtained from as many as 20 injections administered simultaneously. Specificity was demonstrated regardless of the multiplicity of injections.

M. avium organisms were readily transmitted from the inoculated chickens to the calves and one normal chicken. The frequent contamination of feed and water supplies with chicken feces containing viable *M. avium* organisms was considered the major source of infection for the calves and one normal chicken. The importance of excluding chickens from the environment of cattle to prevent sensitization or infection with *M. avium* organisms was reconfirmed in this experiment.

Summary

The sensitivity and specificity of several mycobacterial skin test agents were evaluated in four tuberculous calves. Two calves were inoculated intratracheally with a suspension of *M. bovis* and two calves were infected with *M. avium* by association with tuberculous chickens. Of 100 injections of test agents and four injections of control buffer, 97 responses were compatible with the theory of allergenic specificity.

Healthy calves and chickens were readily infected with *M. avium* by association with tuberculous chickens. The importance of excluding chickens from the environment of cattle is confirmed as a means of preventing nonspecific sensitization and *M. avium* infection of cattle.

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Thoen, C. O., Karlson, A. G. and Ellefson, R. D. Studies on Mycobacteria from Tuberculous Adenitis in Swine. 10th Internatl. Cong. for Microbiol. 1970. (Abstract of published report.)

Mycobacteria (Battey Group) have been isolated from adenitis in swine in various countries. Because such mycobacteria have been isolated from patients in North Central regions of the United States including Minnesota, mycobacteria from swine in this State were studied to determine if Battey bacilli were present.

Cultures of mycobacteria were obtained from 25 of 36 tuberculous lesions in lymph nodes. By animal pathogenicity tests in Japanese quail and in guinea pigs, 24 of the cultures were identified as *Mycobacterium avium.* The one strain not pathogenic for the quail was identified serologically as Watson type (Schaefer). Of the *M. avium* identified, six were Type I and 18 were Type II. By gas chromatography of cell extracts, no consistent difference was found between the fatty acids of the different serotypes including the Watson type. The fatty acids of the *M. avium* appeared to be similar to those of the Battey bacilli from human patients. Histopathologic studies of the lesions from the swine revealed no difference between those caused by the different serotypes. Differences could not be detected by cross sensitivity tests using avian and Battey tuberculin in guinea pigs. It is concluded that in the region of Minnesota, adenitis in swine is caused mainly by *M. avium*.

VESICULAR STOMATITIS

Wright, H. S. Inactivation of Vesicular Stomatitis Virus by Disinfectants. Appl. Microbiol. 19: 96-99. 1970. (Abstract of Published Report).

Twenty-four chemical disinfectants considered to be viricidal were tested. Ten disinfectants were not viricidal for vesicular stomatitis virus within 10 min. at 20° C. when an LD50 titer of $10^{8.5}$ virus units per 0.1 ml. were to be inactivated. Quantitative inactivation experiments were done with acid, alkaline, and a substituted phenolic disinfectant to determine the kinetics of the virus inactivation. Substituted phenolic disinfectants, halogens, and cresylic and hydrochloric acids were viricidal. Basic compounds such as lye and sodium metasilicate were not viricidal.

Jenney, E. W., Hayes, F. A., Brown, C. L. Survey for Vesicular Stomatitis Virus Neutralizing Antibodies in Serums of White-Tailed Deer (Odocoileus virginianus) of the Southeastern United States. Proc. 1970 Ann. Wildlife Dis. Conf. To be published in the Journal of Wildlife Disease. (Abstract of published report.)

New Jersey type vesicular stomatitis (VS) antibodies were found in 14 of 677 deer serums tested by neutralization tests in embryonated chicken eggs. Twelve positive serums were received from Louisiana and two from Georgia. Eight of the positive deer serums from Louisiana were collected in the area of the only reported case of VS during 1967.

Clinical VS has not been diagnosed in the east coast states since 1964. Two positive deer serums were collected on Ossabaw Island, Ga., and three positive serums, one each from a hog, bull, and sheep, were collected from young animals on the island. These findings indicated subclinical infection or a nidus of New Jersey VS in Georgia. The low percentage of New Jersey type VS antibodies in deer and the distribution parallel the low incidence of VS since 1964. No antibodies were present for Indiana type VS virus.

Wright, H. S. Test Method for Determining the Viricidal Activity of Disinfectants Against Vesicular Stomatitis Virus. Appl. Microbiol., 19: 92-95. 1970. (Abstract of published report.)

A test method using embryonating chicken eggs for the evaluation of several types of disinfectants as viricides against vesicular stomatitis virus was developed which gave specific, reproducible results.